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Multiple paternity in a viviparous toad with internal fertilisation

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Abstract

Anurans are renowned for a high diversity of reproductive modes, but less than 1% of species exhibit internal fertilisation followed by viviparity. In the live bearing West African Nimba toad (*Nimbaphrynoides occidentalis*), females produce yolk-poor eggs and internally nourish their young after fertilisation. Birth of fully developed juveniles takes place after nine months. In the present study, we used genetic markers (eight microsatellite loci) to assign the paternity of litters of 12 females comprising on average 9.7 juveniles. In nine out of twelve families (75%) a single sire was sufficient; in three families (25%) more than one sire was necessary to explain the observed genotypes in each family. These findings are backed up with field observations of male resource defence (underground cavities in which mating takes place) as well as coercive mating attempts, suggesting that the observed moderate level of multiple paternity in a species without distinct sperm storage organs is governed by a balance of female mate choice and male reproductive strategies.

Keywords: multiple paternity, internal fertilisation, operational sex ratio, male harassment, Amphibia, *Nimbaphrynoides occidentalis*

Introduction

It is now well established that polyandry is more common in the animal kingdom than originally assumed (Pizzari and Wedell, 2013). For females, advantages of multiple matings include an increased availability of ressources provided by males (Gray, 1997), enhanced fertilisation success (Caspers et al., 2014; Plough et al., 2014), higher genetic heterogeneity (Nichols et al., 2015; Rovelli et al., 2015) and genetic quality of offspring (Bouwman et al., 2006; Byrne and Whiting, 2011; Johnson and Brockmann, 2013). In species with internal fertilisation, polyandry also enables females to excert cryptic choice of fertilising males (for

reviews see Parker and Birkhead, 2013; Simmons, 2005). Negative effects of polyandry for females can include higher predation risk (Arnqvist, 1989) and female injuries during mating (Xavier, 1971), and a higher probability to suffer from sexually transmitted parasites and diseases (Ashby and Gupta, 2013). Males, on the other hand, generally maximise their reproductive success through rather opportunistic and sometimes coercive matings (Arnqvist, 1989; Arnqvist and Kirkpatrick, 2005; Sztatecsny et al., 2006). However, male mating strategies that involve for example territoriality can also reduce the level of polyandry through female monopolisation (e.g.: Arnqvist and Kirkpatrick, 2005).

Apart from differential mating strategies exhibited by each sex, levels of polyandry are also governed by reproductive life histories. Among the classes of vertebrates, amphibians arguably demonstrate the highest diversity of reproductive modes (Duellman and Trueb, 1986; Wells, 2010). Caecilians and caudates are generally characterised by internal fertilisation (Wake, 1993), with sperm storage organs allowing for sperm competition and multiple paternity which are possibly influenced by cryptic female choice (Caspers et al., 2014; Jehle et al., 2007; Kupfer et al., 2008; Rovelli et al., 2015). In anurans, on the other hand, the vast majority of species fertilise their eggs externally (Wake, 1993, 2015a). Polyandry in anurans is common (for a review see Byrne and Roberts, 2012) and male mating tactics such as multiple amplexi (Byrne and Roberts, 1999; Byrne and Whiting, 2011), clutch piracy (Vieites et al., 2004) and possibly the release of stray sperm into aquatic environments (Hase and Shimada, 2014) can lead to multiple paternity within single clutches beyond the control of females.

Internal fertilisation is a prerequisite for viviparity through uterine egg retention until an advanced developmental stage (Wake, 1993, 2015a, 2015b). However, while viviparity is common in caecilians, it is only exhibited by 2.7% of caudates (Buckley, 2012; Wake, 2015a, 2015b). In anurans, only 0.3% of species (17 out of approximately 6600, Frost, 2015) from

five genera are known or assumed to be viviparous, comprising three different viviparous reproductive modes (Haddad and Prado, 2005; Iskandar et al., 2014). One mode is generally termed ovo-viviparous, in which yolk-rich eggs are retained in the uterus and juveniles are born after metamorphosis (Haddad and Prado, 2005; Lamotte and Xavier, 1972; Wake, 1978). This is the viviparous reproductive mode known or assumed for 15 viviparous anuran species, whereas the remaining two modes are each known from only a single anuran species. The second viviparous reproductive mode is generally termed viviparous, or truly viviparous, in which mothers nourish their larvae during gestation and juveniles are born after metamorphosis (Haddad and Prado, 2005; Xavier, 1977, 1986). Only recently the third viviparous reproductive mode was discovered: *Limnonectes larvaepartus* giving birth to tadpoles (Iskandar et al., 2014). In comparison to oviparous species, viviparity is often more costly for females, as clutch sizes are restricted by the available intra-uterine space and locomotion may be reduced (Blackburn, 1999; Shine, 1987; Wourms and Lombardi, 1992). Compared to egg-laying internal fertilisers, cryptic female choice should therefore pose a particular advantage to viviparous species.

The West African Nimba toad (*Nimbaphrynoides occidentalis*) is the only known anuran representative of the truly viviparous reproductive mode (Wake, 2015b; Wells, 2010; Xavier, 1986). Nimba toads mate at the end of the rainy season (September-November), before moving underground until the beginning of the next rainy season in March or April (Lamotte, 1959). After internal fertilisation of small, yolk-poor eggs (~ 500 µm in diameter, Xavier, 1986), females nourish their foetuses (matrotrophy) and give birth to 4-17 fully developed juveniles (pueriparity) after about 9 months (Lamotte, 1959). At a longevity of 3-5 years (Castanet et al., 2000), lifetime reproductive output per female is low.

In the present paper we focus on the Nimba toad to, for the first time, elucidate patterns of paternity in a viviparous anuran with internal fertilisation. More specifically, we link our

findings from genetic parentage analyses of litters derived from known females with behavioural field observations of male fighting and mating attempts. Together with presenting data on operational sex ratios, we discuss whether internal fertilisation in the Nimba toad leads to a genetic mating system which can be more controlled by females compared to the external fertilisation which is exhibited by the vast majority of anurans.

Materials and Methods

Field work

The study was conducted in the high altitude grasslands of the Guinean Nimba Mountains, West Africa. The area is characterised by a dry season from December to March and a rainy season from April to November, a seasonality which has a strong influence on Nimba toad abundance and sex ratios (Lamotte, 1959). A male-biased operational sex ratio (OSR) was previously hypothesised to be the main driver for anuran polyandry (Byrne and Roberts, 2012; Hase and Shimada, 2014; Lodé et al., 2005; Sztatecsny et al., 2006). Therefore, we estimated the average OSR per calendar week within the rainy season based on annual monitoring data from 2007 to 2014. We included 333 areas of 5 x 5 m in size at a search effort of 90 person minutes (for more details see Hillers et al., 2008; Sandberger-Loua et al., 2016). Weekly OSRs were fitted to an additive generalised model using the mgcv package in R (Wood, 2011).

We collected 12 gestating females in the field, and kept them singly in plastic terraria about 5 km from the capture location, feeding them with small flying insects and termites. Humidity levels were kept high through moistened polyurethane foam, and the temperature was kept at levels representing their natural environment (20-25°C). All females gave birth between three and 10 days after capture. After birth, we clipped a tip of the second toe from the mother and all offspring (following procedures described in (Grafe et al., 2011)), to store in 98% ethanol

for DNA analysis. All toads (mothers and juveniles) appeared in good condition after this procedure and were released where the mother had been caught.

Development of microsatellite loci for N. occidentalis

We used up to eight newly developed microsatellite loci to determine the number of fathers in each litter. For development of a genomic library, DNA was extracted from thigh muscles of six individuals using Puregene DNA Purification Kit (Gentra Systems) according to the manufacturer's recommendations. GENterprise Genomics (Mainz, Germany; http://genterprise.de) was contracted to develop a repeat-enriched library. Twenty-seven sequences containing more than eight repeats and sufficient flanking regions were chosen to design PCR primers using Primer3 (Rozen and Skaletsky, 2000). Eight loci proved polymorphic and unambiguously scorable based on an initial test with 4 individuals and subsequent characterisation based in a further 40 individuals. Microsatellite sequences were deposited in Genbank.

Genotyping

DNA for the paternity analysis was extracted using the Roche PCR template preparation kit according to the manufacturer's recommendations. PCRs were performed using a 12.5 µl PCR reaction volume containing 1 ×PCR-buffer, 2 mM $MgCl_2$, 160 µM dNTPs, 2.5 µM of each primer (forward primer labelled with fluorescent IR-700 or IR-800 dye by Licor), 0.5 U of Taq DNA polymerase (New England BioLabs) and 1 µl of 1:10 diluted template DNA. All loci were amplified on a 2720 Thermal Cycler (Applied Biosystems, version 2.09). Loci G07 and D03 were amplified using a fixed annealing temperature of 57°C, and a touchdown program was applied to all other loci (Nocc4, A09, C05, C10, E06 and F03). PCR conditions for the two protocols were as follows: 57°C: 3 min at 94°C, 35 cycles with 30 sec. each at

94°C, 57°C and 72°C, followed by 20 min at 72°C; touchdown: 5 min at 94°C, 10 cycles with annealing temperature decreasing 0.5°C per cycle from 63°-57°C, with 30 sec. each at 94°C, annealing temperature and at 72°C, followed by 25 cycles with 30 sec. each at 94°C, 55°C and 72°C, followed by 7 min elongation at 72°C. Allele lengths were analysed with SAGA^{GT} (LICOR). Primer characteristics are given in Table 1. To minimise scoring errors, every sample was amplified at least twice for each locus.

Paternity analyses

We used two complementary approaches to estimate the minimum (GERUD) and the most likely (COLONY) number of sires in each litter, respectively. In a first step, we applied the maximum likelihood approach implemented in COLONY2 (Jones and Wang, 2010) assuming a large error rate (10%) to identify potential genotyping errors. The following specifications were used: the maternal genotype was known, no candidate fathers included, we gave no known population allele frequency, we used a sib-ship size prior and two runs of medium length. Individuals whose alleles were classified as typing errors (0.4% of alleles or one allele in 3.4% of individuals) were genotyped a third time and in all cases the allele sizes were confirmed. Final results from COLONY2 runs were subsequently derived with an assumed error rate of zero (all other specifications as described above), and repeated a second time with the inclusion of population-wide allele frequencies. Population-wide allele frequencies were derived from a dataset from 600 adult toads (Sandberger-Loua et al., unpublished). As a second approach, we applied GERUD2.0 (Jones, 2005) to estimate the minimum number of fathers to explain the observed genotypes. Because GERUD2.0 does not allow for missing data, we had to alternatively exclude 17% of individuals or reduce the number of markers used. The statistical power was assessed by running GERUD2.0sim (Jones, 2005) 10 times with 1000 iterations, assuming that the mother's genotype is known,

that one of 10 offspring is sired by a second male, and that either four or five loci are available, based on the population wide allele frequencies. In all 10 runs, an additional father was recognised in > 99% of iterations, demonstrating that only a subset of available loci is sufficient for reliable paternity detection. We considered litters to contain multiple paternity when GERUD and COLONY independently identified multiple fathers, and when the number of most probable sires determined by COLONY was identical or larger than the minimum number of fathers estimated by GERUD. The effective mating frequency (m_e), a quantity which takes the actual paternity of contributing males into account, was calculated as $m_e = (1/\Sigma pi^2)$**,** where *pi* is the proportion of offspring in a clutch sired by male *i* (Starr, 1984).

Results

The 12 Nimba toad females gave birth to an average of 9.7 young each (range: 4–16; total 116). We genotyped 5-7 loci for each individual and included 117 individuals in the paternity analysis (12 mothers and 105 offspring, 11 young had to be discarded due to poor DNA quality, Table 2). The microsatellite markers proved highly polymorphic, comprising on average 14 alleles per locus (range: 7–19 alleles). All offspring could be unambiguously assigned to their mothers, and in nine out of twelve families a single sire was sufficient to explain the observed genotypes in each family. Three families contained multiple paternity as identified by both GERUD and COLONY, with the most successful male siring between 55-70% of offspring (effective mating frequency, m_e: 1.72–1.98; Table 2). The same results were obtained when considering population allele frequencies (data not shown). However, whereas GERUD identified two fathers in all three cases, COLONY identified two families with two fathers and one family with three fathers. This discrepancy in sire number is due to GERUD and COLONY identifying the minimum and the most likely number of fathers, respectively.

In total, 7.6% (COLONY) and 8.6% (GERUD) of juveniles were estimated to be fathered by a secondary sire.

The operational sex ratio (OSR) significantly progressed from a female bias at the beginning of the mating season, increasing to a male bias towards the end of the mating season (GAM: estimate: 0.55, $t = 10.17$, $p < 0.001$, adjusted r^2: 0.59, Fig1).

Discussion

This is the first study examining genetic paternity in an anuran with internal fertilisation, demonstrating the occurrence of multiple sires at a moderate proportion of examined litters. In line with a high diversity of reproductive modes, previous studies on frequencies of multiple paternity in externally fertilising anurans revealed a range spanning from 0% (Brown et al., 2010) to over 90% (Byrne and Whiting, 2011). For internally fertilising caudates, multiple paternity was reported to range between 38% (Caspers et al., 2014) and 96% of clutches (Adams et al., 2005). Our results for the Nimba toad (25%) are lower than those reported for caudates, and in the lower part of the anuran range. Polyandry allows for fertility assurance, sperm competition, and possibly cryptic female choice (Birkhead, 1998). In Nimba toads fertilisation rates have previously shown to be high (on average 90%, Lamotte et al., 1964). It is currently unknown whether in Nimba toads mating with high quality males results in more eggs being ovulated (as observed e.g. in carnivore mammals, Larivière and Ferguson, 2003). In contrast to other bufonids, Nimba toads engage in a lumbal amplexus through which males clasp females in the region of their ovaries (Xavier, 1971), possibly inducing or facilitating ovulation. However, females kept without males ovulate and develop a pseudo-gestation (Xavier, 1974), and atresia of follicles should occur if not all mature eggs are ovulated due to missing induction, but is very rare (Xavier, 1971; Xavier et

al., 1970). Taken together, it seems likely that other factors than increased fertilisation success are the main drivers for polyandry.

In contrast to caudates, internally fertilising anurans have no apparent sperm storage organs (Wake, 2015a). In Nimba toads due to the development of a pseudo-gestation without mating as well in older females (> 2 years, Xavier, 1974), inter-season sperm storage can be excluded. Nevertheless, short-term sperm storage (hours to days) can still allow for sperm competition and cryptic female choice (Orr and Brennan, 2015). Relative testes size is generally correlated with the level of sperm competition (e.g. Dziminski et al., 2010). Accounting for about 0.4% of body weight, (Gavaud, 1976; own data), Nimba toad males have normal sized testes comparable to other anurans without evidence for elevated sperm competition (Kusano et al., 1991). Additionally, despite a large sample size of investigated females, no accumulation of sperm was found in their reproductive organs (Xavier 1971). Considering that amplexus in Nimba toads can last for more than one day (Xavier, 1971), cryptic female choice is only possible if spermatozoa remain viable for longer time periods than mating duration. Another possibility for females to favour offspring from high quality males, is to vary resource allocation. Gestating Nimba toad females secrete mucoproteins from uterine epithelial cells into the uterus cavity, on which developing young are orally feeding (Vilter and Lugand, 1959; Xavier, 1971, 1977). It is therefore likely difficult for females to favour offspring sired by particular males. Hence, so far no indication for increased sperm competition was recorded for males, nor sound indication for sperm longevity or sperm storage in females, nor mechanisms enabling females to vary the provided resources. Additionally, the prolonged amplexus is costly for females, as they are injured by the tight grip and the nuptial spines of males (Xavier, 1971). Taken together, these observations indicate that pre-mating female choice might be more important than post-mating (cryptic) female choice in conjunction with sperm competition.

In externally fertilising anurans, male display territories or defence of resources such as breeding sites can facilitate female choice (Chen et al., 2011; Hudson and Fu, 2013; Lodé and Lesbarrères, 2004). Our field observations suggest similar phenomena in Nimba toads. Males defend entrances to underground cavities in which Nimba toads reside during the dry season and in which matings are assumed to take place (Angel and Lamotte, 1947, see Online Resource 1 for a field observation). To enter these cavities at the end of the rainy season, females have to pass the guarding males, providing them an opportunity for mating. Individual males can be observed over several weeks around the same cavity entrances which they defend against other males (Sandberger-Loua, personal observation). Males also engage in antagonistic behaviour accompanied by calling and often physical combat (see Online Resource 2). Unfortunately, we were not able to sample DNA in the field from males to test the hypothesis that individuals winning combats at entrances to dormancy cavities sire the majority of offspring in given litters. Nevertheless, for externally fertilising anurans, within-clutch multiple paternity appears to be lower for territorial species (0-29%: Brown et al., 2010; Chen et al., 2011; Hudson and Fu, 2013; Knopp and Merilä, 2009) compared to non-territorial species (30 - > 90%: Byrne and Roberts, 1999; Byrne and Whiting, 2011; Hase and Shimada, 2014; Sztatecsny et al., 2006; Vieites et al., 2004). Despite reporting on an internal fertiliser, our results are in line with the proportions of polyandry reported for other anurans with territorial males, suggesting that Nimba toad females may not have more control over genetic sires than females of species with external fertilisation.

In reptiles, high levels of polyandry may mainly arise from the combination of high male benefits and low female cost from frequent mating (e.g.reviewed in Uller and Olsson, 2008). Similarly, for several anurans, male harassment coupled with a male-biased OSR was found to be the main driver for polyandry (Byrne and Roberts, 1999; Hase and Shimada, 2014; Sztatecsny et al., 2006; Zhao et al., 2016). During the breeding season, female Nimba toads

are most commonly found hiding in small shelters (Sandberger-Loua, personal observation), probably avoiding undesired matings and harassment by males. Additionally, possibly providing females the opportunity to observe males and judge on their, or their cavity's, quality. Males target both single females as well as amplected pairs for mating attempts, trying to dislodge the male in the latter case (Online Resource 3). Due to the viviparous reproductive mode, clutch sizes are very small and a female's value hence, presumably very high. This may justify the high male effort to defend territories and harass females and the taken increased predation risk due to the calling and fighting activity. Mainly cavities and to a lesser extend females are defended by males, possibly indicating a high value of dormancy cavities for females and multiple paternity may arise if females change dormancy sites. Interestingly, females also appear to physically resist mating with specific males by dislodging them from their back (Online Resource 4). In externally fertilising anurans with male harassment, polyandry levels similar to Nimba toads were observed (19-30%: Lodé et al., 2005; Sztatecsny et al., 2006). The level of male harassment is likely related to the OSR, which becomes male-biased towards the end of the mating season. We however lack the information to test the hypothesis that the documented cases of multiple paternity stem from matings at the end of the reproductive period. Future studies should examine the effect of a male-biased OSR, female efficiency of dislodging males and the existence of mate order effects on the proportions of multiply sired clutches. Due to the internal fertilisation, successfully dislodging males may give Nimba toad females more control over genetic sires than females of species with external fertilisation.

Conclusion:

Representing the first study of polyandry in an anuran with internal fertilisation, we reported moderate proportions of multiple paternity in the viviparous Nimba toad. High levels of

sperm competition and cryptic female choice are unlikely for example due to the lack of morphological adaptations, and male territoriality in combination with matings which are costly for females. This suggests that pre-mating female choice is likely more important than cryptic female choice to determine the observed genetic mating system in Nimba toads. Taken together, we assume that the observed frequency of multiple paternity is caused by a combination of female choice and male harassment. Because such effects can also be observed in anurans with external fertilisation, they are not indicative for an increased female control over paternity in Nimba toads compared to other anurans. Nevertheless, the ability for Nimba toad females to physically resist male mating attempts in combination with internal fertilisation may give females more control over the genetic mating system compared to the majority of externally fertilising anurans.

Compliance with Ethical Standards

The study was funded by the Société des Mines de Fer de Guinée (SMFG), but the company had no influence on study design, data collection, data analyses and interpretation, writing of the manuscript and in the decision to submit the paper for publication, thus the authors declare that they have no conflict of interests.

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All work complies with the guidelines for the use of live amphibians and reptiles in research compiled by the American Society of Ichthyologists and Herpetologists (ASIH), The Herpetologists' League (HL) and the Society for the Study of Amphibians and Reptiles (SSAR), as well as to the IUCN policy statement on research involving species at risk of extinction. The Ministère de l'Enseignement Superieur et de la Recherche Scientifique (MESRS) and the Direction Nationale de la Recherche Scientifique et Technique (DNRST) granted research permits (N°085/DNPN/2007, N°103/DNRSIT/DN,

N°095/MENSRS/DNRST; N°091/MESRS/DNRST/2009; N°121/MESRS/DNRST/2010; N°177/MESRS/DNRST/2011, N°027/MESRS/DNRST/2012, N°061/DNRSIT/DN and N°020/MESRS/DNRSIT/2014). The authorities from the Ministère de l'Environement et du Development durable, Conakry and the Bundesamt für Naturschutz, Bonn granted CITES export (N°00314,N°00492) and import permits (E-3117; E-4074), respectively.

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Online Resources:

Online Resource 1: Video1.avi, defending cavity entrance: shows a behavioural observation of a male defending a cavity entrance against another male.

Online Resource 2: Video2.avi, antagonistic behaviours: shows behavioural observations of male antagonistic behaviours as aggressive calling and fighting.

Online Resource 3: Video3.avi: male harassment: shows a male harassing a female, as well as a male trying to dislodge an amplected male.

Online Resource 4: Video4.avi: female dislodges male: gives an example of a female dislodging an amplected male from her back.

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514 Figure caption

515 Fig 1 Operational sex ratio for Nimba toads within the rainy season. Shown is the average
516 operation sex ratio (OSR) per calendar week, based on annual monitoring data in those areas
517 where females for this study were captured; data recorded between 2007 and 2014 (based on
518 333 squares (5x5m) examined for Nimba toads). The mating season is indicated in grey. The
519 line shows the predictions of the OSR of an additive generalised model. The horizontal line
520 indicates and equal OSR. The inset figure in the upper left shows a brightly coloured male,
521 the inset in the lower right a pair in amplexus. The graphic was produced in R, the inset
522 photographs added in Illustrator.